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Original Article

Does hepatitis C virus play a role in breast and colon cancer?

Ahmed Roshdi Hamed Ahmed, Afaf Taha E. AlNashar, Ghada Ahmed Askar, Amira A. Abdelnaby

*Pathology Deparment, Faculty of Medicine, Sohag University Egypt

Abstract

Background: Hepatitis C virus (HCV) is an important cause of hepatocellular carcinoma (HCC) worldwide. The highest incidence of HCV is in Egypt, where some epithelial cancer incidence has increased. May be there is a link between HCV infection and carcinogenesis of non-hepatic epithelial tumors.

Objectives: In this study, we test the presence of HCV antigen in non-hepatic epithelial tumors as breast and colon cancers.

Patients and methods: Fifty-five tumors; 23 colon and 32 breast cancers were included in this study. All patients were HCV seropositive. All tumors were examined immunohistochemically (IHC) using HCV core antibody and HCV Core + NS3 + NS4 antibodies (Cocktail of HCV proteins) to evaluate the diagnosis and to assess the presence or absence of HCV antigen.

Result: It was found that 52 of the studied tumors showed HCV core protein expression including; 22 and 3، of colon and breast cancers respectively. The three negative tumors included one colon cancer case and two breast cancer cases. All investigated tumors showed expression of Cocktail of HCV proteins with different intensities. The expression of both HCV proteins was either nuclear, cytoplasmic or nucleocytoplasmic and granular pattern.

Conclusion: Most breast and colon tumors expressed HCV antigens, suggesting that HCV infection may play a role in the carcinogenesis of these tumors.

Keywords: Hepatitis C virus, colon cancer, breast cancer, immunohistochemistry.

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Corresponding Author: **Ghada Ahmed Askar** **E.mail**: ghada-med@hotmail.com

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Abbreviations: HCV; Hepatitis C virus, **HCC;** hepatocellular carcinoma. **IHC;** immunohistochemically or immunohistochemical, **H&E;** Hematoxylin and eosin, **Cocktail of HCV proteins;** HCV core+NS3_NS4.

Introduction

HCV is a major health problem as it represents an important cause of morbidity and mortality. The prevalence of HCV is 2% worldwide. HCV is one of the most important causes of post transfusion hepatitis, causes various extrahepatic manifestation in addition to the hepatic manifestation.[15] The oncogenic potential of HCV is well established for HCC, also it has been thought that our virus play a role in the development of other extrahepatic tumors, especially that HCV has been isolated from many epithelial tissue.[16] In Egypt, the incidence of malignancy affecting some organs is elevated as breast and colon cancers, and the incidence of HCV virus in our community is already high. In this study we tried to find if there is a relationship between viral infection and development of tumors of these organs. This hypothesis is also brought up by Fiorino and his colleagues that HCV infection is correlated with extrahepatic malignancy in areas where there is high prevalence for HCV.[7] In our study we use two HCV proteins; HCV core protein which is a famous protein commonly used for HCV detection and Cocktail proteins (HCV core+NS3+NS4) which represent a group of HCV proteins to ensure the detection of the virus within the tissue.

Patients and methods

We performed a retrospective study on 55 formalin fixed paraffin-embedded tissue specimens from HCV serologically positive patients, including 23 and 32 tumors diagnosed as colon cancer and breast cancer respectively in Sohag Cancer Institute, in period from January 2018 to December 2020. Clinical data were obtained from patients' clinical files including: patients' age, sex and positive PCR for HCV. Hematoxylin and eosin (H&E) stained sections were examined to evaluate the diagnosis and tumor grading. For statistical purpose and because of different staging system for each organ, stages of the tumors were combined to deduce two categories; early stage and advanced stage.

Ethical consent:

Ethical approval was obtained from Sohag University Ethical Committee. The research was conducted in compliance with the ethical guidelines set by the World Medical Association's Declaration of Helsinki for studies involving human participants. IHC technique was used to detect HCV core protein and cocktail of HCV proteins in colon and breast cancers.

All submitted samples were formalin-fixed, paraffin-embedded tissue material, cut by a microtome at 5μm thickness and picked up onto poly-L-lysine coated slides and dried overnight at room temperature. After dewaxing and rehydration, tissue sections were pretreated in citrate buffer (pH 6.0) using microwave at 70°C for 20 minutes, followed by blocking of endogenous peroxidase. One set tissue sections were then incubated with mouse HCV core monoclonal antibody; antihepatitis C virus, clone H6-29 (Catalog number; Cat # GTX64113, Gene Tex International, Corporation, Fremont, USA); 1:400 dilution overnight at 4°C. Avidin-biotin peroxidase complex detection system from LabVision Corporation was applied with 3,3diaminobenzidine as chromogen (LabVision, Fremont, USA). Another set tissue sections were incubated with goat polyclonal antihepatitis C virus antibody (HCV core+NS3+NS4), (Cat # GTX40324, Gene Tex International, Corporation, Fremont, USA); 1:300 dilution overnight at 4°C. Rabbit antigoat polyclonal Horseradish peroxidase conjugated secondary antibody (Cat # A16136, Thermo Fisher Scientific, USA) was used at 1: 250 dilution for 30 minutes, with 3,3 diaminobenzidine as chromogen (LabVision, Fremont, USA). HCC samples positive for HCV core protein and Cocktail proteins were used as positive controls for IHC staining.

HCV core and Cocktail proteins expression appeared as brownish granular cytoplasmic and nuclear staining. Semi-quantitation of cytoplasmic HCV core and Cocktail proteins immunoreactivities were assessed. Both the intensity of immunereaction and the percentage of positive cells were

considered in the final score. Evaluation of the percentage of positive cells was equal to the mean percentage of positive cells in 4 high power fields; HPFs). The intensity of immunoreaction was weighed as follow: 0= negative cells, 1= mild intensity, 2= moderate intensity and 3= strong intensity. [6]

Results were statistically analyzed using Statistical Package for Social Sciences 16 (SPSS 16) for Windows. Pearson's, Chi-Square test, T test and Mann-Whitney were used to evaluate Statistical significance of various parameters, and $p<0.05$ was taken as the level of significance.

Results

The age of investigated cases was ranged from 30 to 85 years old with mean ± SD was (59.73± 11.3) and median was 60. The range of age of colon cancer cases was 35 to 85 with mean ± SD was 62.52 ± 10.61 and median was 62. Breast cancer cases have age range 30 to 83 with mean ± SD was 57.7 ± 11.5 and median was 60. There was no significant association between the type of tumor and the age. The frequency of male and female in colon tumors were nearly equal. The majority of colon cancer tumors were adenocarcinoma (21 tumors), one tumor was melanoma and another tumor was gastrointestinal stromal tumor; GIST as the study include all types of colon cancers.

The majority of breast cancer tumors were infiltrating ductal carcinoma; IDC (28 tumors), three tumors were invasive lobular carcinoma and one tumor was metaplastic carcinoma.

The size of tumors ranged from 1 to 13 cm with mean ± SD of 4 ± 2.4 and median of 3 cm. In colon cancer tumors, the range of size was 1 to13 cm with mean ± SD of 1.7 ± 0.5 and median of 2, and for breast cancer cases was 1 to 7 cm with mean ± SD of 1.34 ± 0.5 and median of 1. Statistical analysis revealed significant relation between type of tumor and size of tumor (Mann-Whitney=0.025).

Colon cancer was graded histologically as grade I, grade II and grade III tumors, in 3, 15, 5 cases respectively. Breast cancer cases were graded as Grade II in 26 cases and grade III in 6 cases. Most of colon tumors revealed no lymph node metastasis unlike breast cancer cases at which the highest percentage (56%) of tumors had lymph node metastasis with no significant relation between type of tumor and lymph node status.

The frequency of colon cancer cases with advanced stage is significantly higher than those with early stage, while the opposite occurs in breast cancer cases with no significant relation between type of tumor organ and tumor staging. Regarding lymphovascular invasion, it had significant association with tumor type (Chi-square=0.0001). Most of colon and breast tumors had mild tumor infiltrating lymphocytes as shown in(table1)

Table (1): Clinico-pathological parameters in the studied tumors of both organs

	Colon	Breast	Total
Sex			
• Male	11	1	12
• Female	12	31	43
Tumor type	23	32	55
Tumor grade			
• Grade I	3	0	3
• Grade II	15	26	41
• Grade III	5	6	11
Lymph node status			
• Positive node metastasis	6	18	24
• Negative lymph node metastasis	13	14	27
Tumor stage	2	25	27
• Early stage	17	7	24
• Advanced stage			
Lympho-vascular invasion			
• Detected L.V invasion	3	8	11
• No detected L.V invasion	16	24	40
Tumor infiltrating lymphocytes			
• Mild	12	28	40
• Moderate	10	4	14
• Strong	1	0	1

Expression of HCV core molecule:

We detected HCV core protein by IHC in the tissues of 52 studied cases, including 22 cases of colon cancer and 30 cases of breast cancer and the staining pattern in these tumors was either nuclear in 2 tumors, cytoplasmic in 41 tumors or nucleocytoplasmic in 9 tumors.

H score of all investigated tumors ranged from 0 to 290. The range of H score of colon tumors was 0 to 285, the range of H score of breast tumors was 0 to 290 (figure 1). The mean±SD of H score of HCV core molecule in colon tumors was 134.4±72.4, and in breast tumors was 122.5±84.3 (table 2).

Expression of Cocktail of HCV molecules:

Cocktail of HCV proteins was detected in tissues of all investigated cases and the staining pattern in these tumors was either nuclear in one tumor, cytoplasmic in 44 tumors or nucleocytoplasmic in 10 tumors.

H score of all investigated tumors ranged from 8 to 300. The range of H score in colon tumors was 29 to 300, the range of H score of breast tumors was 8 to 242 (figure 2). The mean±SD of H score of Cocktail of HCV proteins in colon tumors was 122.8±71.7, and in breast tumors was 122.3±66.1 (table 2).

Table (2): Mean± SD, median, minimum and maximum of HCV core and Cocktail of HCV proteins

	HCV core molecule	**Cocktail molecule**
Mean	127.45	122.49
Standard deviation	79	67.8
Median	123	113
Minimum	0	8
Maximum	290	300

Regarding colon cancer, there was no significant association in the expressions of either HCV core antigen or Cocktail of HCV proteins and most clinico-pathological parameters (table 3). Regarding breast cancer, there was no significant association between the expression of either HCV core or Cocktail of HCV proteins and clinic-pathological parameters (table 4). The relationship between the score of HCV core and Cocktail of HCV proteins and clinic-pathological parameters of colon and breast cancers is summarized in(table 5).

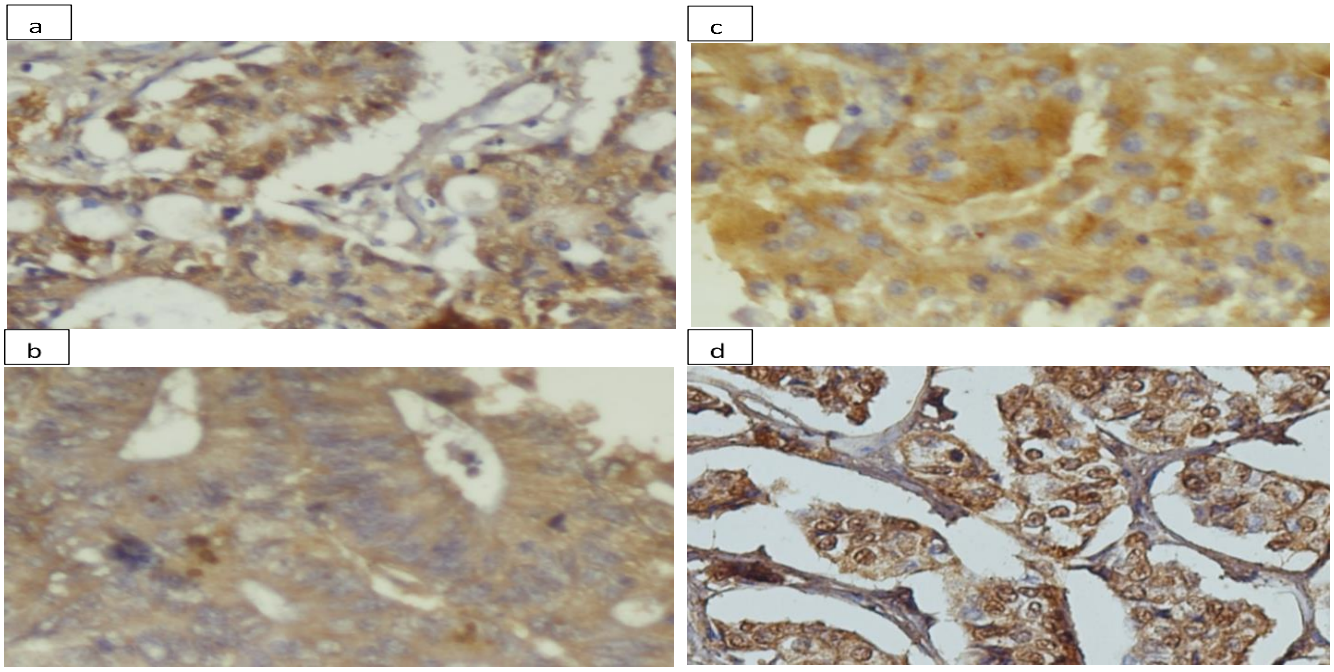

Figure. 1. a: Expression of HCV core molecule in colon adenocarcinoma with nuclear and cytoplasmic pattern (H score is 198/300, x400). b: Expression of HCV core molecule in colon adenocarcinoma with cytoplasmic pattern (H score is 200/300, x400). c: Expression of HCV core molecule in infiltrating ductal carcinoma with cytoplasmic pattern (H score is185/300, x400). d: Expression of HCV core molecule in infiltrating ductal carcinoma with nuclear and cytoplasmic pattern (H score is174/300, x400).

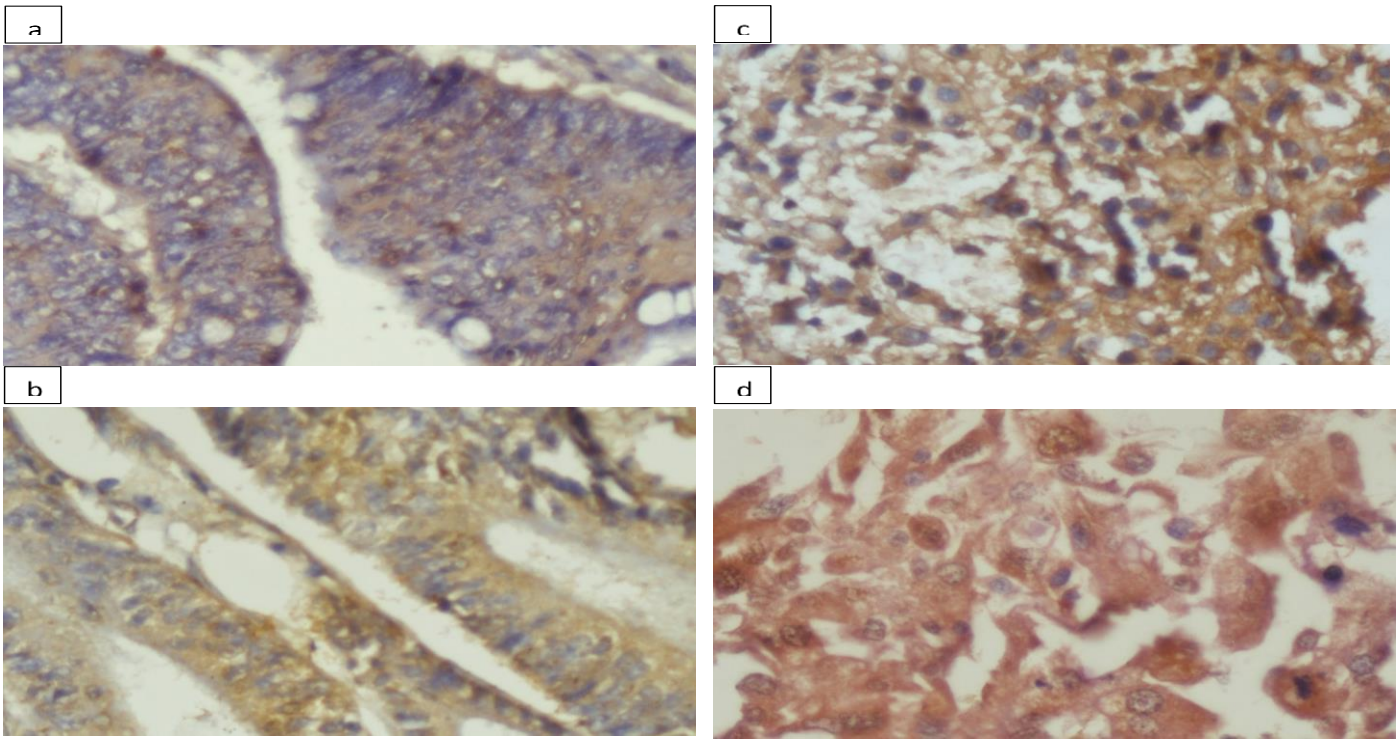

Figure. 2. a: Expression of Cocktail of HCV proteins in colon adenocarcinoma with cytoplasmic pattern (H score is 2 29/300, x400). b: Expression of Cocktail of HCV proteins in colon adenocarcinoma with nuclear and cytoplasmic pattern (H score is 109/300, x400). c: Expression of Cocktail of HCV proteins in invasive lobular carcinoma with cytoplasmic pattern (H score is 142/300, x400). d: Expression of Cocktail of HCV proteins in infiltrating ductal carcinoma with nuclear and cytoplasmic pattern (H score is 235/300, x400).

Table (3): Expression HCV core molecule and Cocktail of HCV proteins in relation to clinico-pathological parameters in colon cancer

Clinico-pathological variables	Number	Mean±SD (Median) H score of HCV core molecule	P value =	Mean±SD (Median) H score Cocktail of HCV proteins	P value =
Age	7				
• <60 years old	16	135.4±81.3 (111)	0.965	158±101.4 (168)	0.121
• >60 years old		133.9±71 (129.5)		107.4±50.9 (106)	
Sex	11				0.362
• Male	12	128.5±59 (123)	0.720	108.2±32.4 (112)	
• Female		139.8±85.1 (119)		136.2±94.4 (123.5)	
Tumor size	7				0.142
• <4 cm	16	88.7±60.2 (81)	0.042	89.3±49.3 (86)	
• >4 cm		154.4±69.5 (131)		137.4±76.2 (119.5)	
Tumor grade	3				0.898
• G1	20	54.7±62.6 (41)	0.037	117.7±44.1 (99)	
• G2,3		146.4±67.1 (129.5)		123.5±75.7 (112)	
Lymph node status					0.856
• N0	13	137.9±80.8 (130)	0.511	132.2±76.4 (112)	
• N1,2,3	6	163±62.5 (162.5)		125±68.7 (132)	
Tumor stage	2				0.849
• T1,2	17	165±49.5 (165)	0.712	120.5±28.9 (120.5)	
• T3,4		143.5±77.9 (129)		131.2±76.1 (112)	
Lymphovascular invasion					
• Yes	3	160.3±66.9 (198)	0.725	143±140.5 (100)	0.746
• No	16	143.1±77.8 (129.5)		127.9±59.8 (113.5)	
Tumor infiltrating lymphocytes					
• Mild	12	124.3±84.9 (89)	0.621	111.3±60.8 (100)	0.340
• Moderate	10	140.3±58.2 (129.5)		126.6±82.1 (134)	
• Strong	1				

Table (4): Expression HCV core molecule and Cocktail of HCV proteins in relation to clinico-pathological parameters in breast cancer

Clinico-pathological variables	Number	Mean±SD (Median) H score of HCV core molecule	P value =	Mean±SD (Median) H score Cocktail of HCV proteins	P value =
Age					
• <60 years old	14	135.4±94 (154)	0.455	120.7±64.2 (105.5)	0.908
• >60 years old	18	112.4±77.1 (115.5)		123.5±69.4 (126.5)	
Tumor size					
• <4 cm	21	118.8±89.2 (121)	0.744	130.3±59.7 (117)	0.353
• >4 cm	11	129.4±77.4 (172)		107±77.8 (117)	
Tumor grade					0.966
• G1	0	0	0.868		
• G2	26	123.7±87.7 (127.5)		122±69 (115)	
• G3	6	117.2±74.3 (139.5)		123.3±57.3 (137.5)	
Lymph node status	14				
• N0	18	113.1±89.2 (115.5)	0.586	127.6±67.9 (113)	0.693
• N1,2,3		129.8±82.1 (154)		118±66.4 (119.5)	
Tumor stage	25				
• T1,2	7	122.5±81.8 (122)	0.999	131.8±56.4 (122)	0.126
• T3,4		122.4±99.5 (172)		88.3±90.3 (47)	
Lymphovascular invasion					
• Yes	8	142.7±87.9 (180.5)	0.441	117±80.1 (129.5)	0.799
• No	24	115.7±83.8 (116)		124±62.6 (115)	
Tumor infiltrating lymphocytes					
• Mild	28	119.5±88.7 (127.5)	0.606	127.3±67.4 (120.5)	0.261
• Moderate	4	143.3±42.9 (131.5)		87±51.8 (108)	
• Strong	0	0			

Table (5): Score of HCV core molecule and Cocktail of HCV proteins in relation to clinico- pathological parameters in breast cancer

Clinico-pathological variables	Number	Mean±SD (Median) H score of HCV core molecule	P value =	Mean±SD (Median) H score Cocktail of HCV proteins	P value =
Age	21	135.4±87.9 (133)	0.564		
• <60 years old	34	122.6±73.98 (121.5)		133.14±78 (113)	٠.944
• >60 years old				115.9±61 (113.5)	
Sex	12	132.5±57.9 (126.5)	0.805		
• Male	43	126.1±84.5 (122)		113.2±35.4 (112)	0.595
• Female				125.1±74.6 (117)	
Tumor size	28	111.32±82.99 (108.5)	0.124		
• <4 cm	27	144.2±72.5 (132)		120±59.2 (111)	0.٧87
• >4 cm				125±76.9 (117)	
Tumor grade	3	54.7±62.6 (41)	0.101		
• G1	52	131.7±78.3 (129.5)		117.7±44.1 (99)	0.901
• G2,3				122.8±69.3 (114)	
Lymph node status	27	125±84.6 (121)	0.570		
• N0	24	138.1±77.7 (154)		129.9±70.7 (112)	0.608
• N1,2,3				120±65.5 (119.5)	
Tumor stage	27	125.6±80 (130)	0.610		
• T1,2	24	137.4±83.1 (130.5)		131±54.6 (122)	0.525
• T3,4				118.7±81 (112)	
Lymphovascular invasion					
• Yes	11	147.6±79.9 (185)	0.454	120.1±92.7 (117)	0.952
• No	40	126.7±81.6 (126)		125.5±60.8 (114)	
Tumor infiltrating lymphocytes					
• Mild	40	120.9±86.6 (129.5)	0.495	122.5±65 (112.5)	0.321
• Moderate	14	141.1±52.6 (129.5)		115.3±75.1 (115.5)	
• Strong	1	-		-	

Correlation between HCV core and Cocktail of HCV proteins:

Statistical analysis revealed significant correlation between HCV core and Cocktail HCV proteins (Pearson correlation coefficient test, p= 0.047). The following charts revealed the correlation between both HCV core and Cocktail of HCV proteins (graph 3a) and between each one of them in both organs (graph 3b, c).

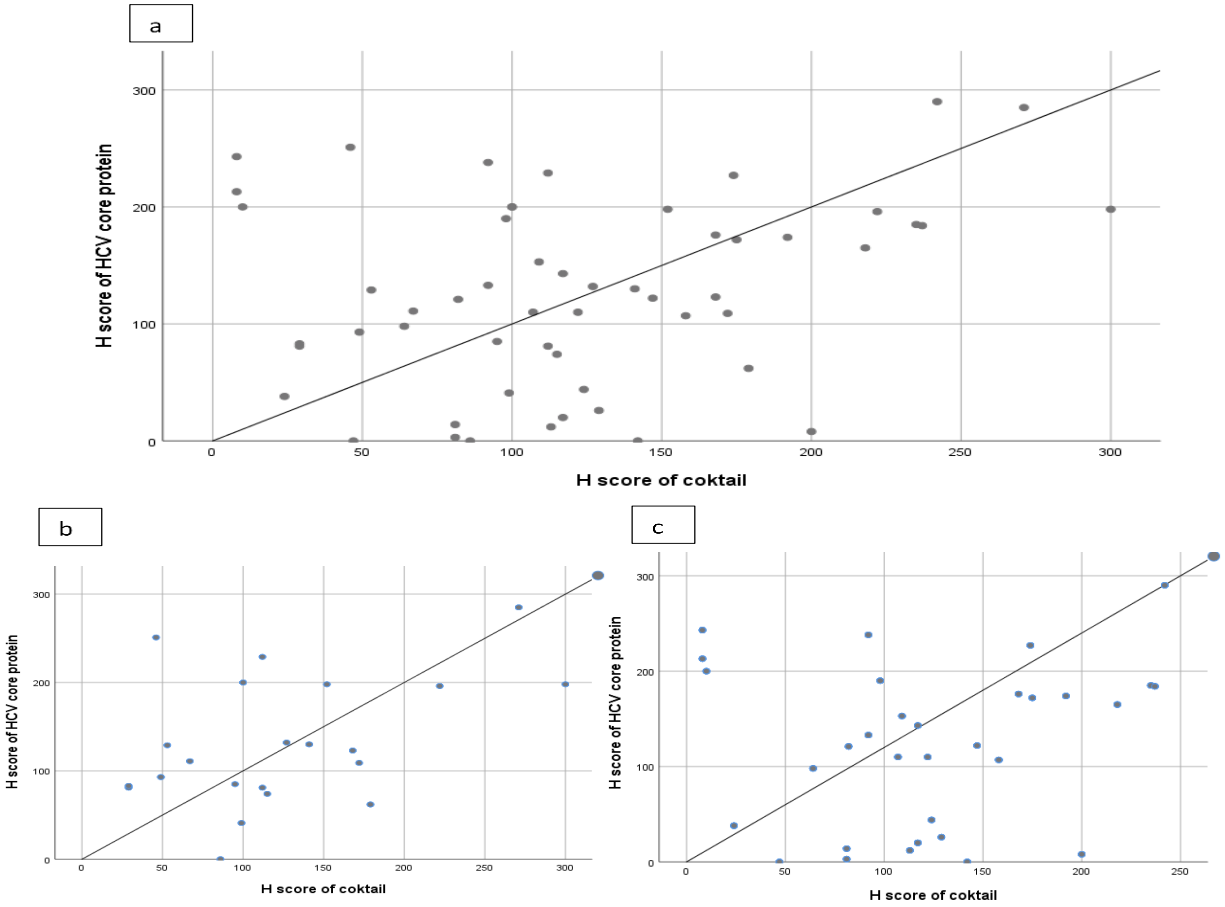

Figure. 3. a: Correlation of HCV core and Cocktail of HCV proteins. b: Correlation of HCV core and Cocktail of HCV proteins in colon cases. c: Correlation of HCV core and Cocktail of HCV proteins in breast cases.

Discussion

In the current study, we adopted the IHC technique for detection of HCV proteins; using HCV core monoclonal IgG antibody and HCV core+NS3+NS4 polyclonal IgG antibody, repressenting structural, and non-structural viral proteins. The pattern of staining is granular cytoplasmic, but few cases have nuclear staining and most of them have nuclear and cytoplasmic pattern which is the same as patterns described by Rullier. [17]

The relation between viral infection and carcinogenesis is well established including the role of EBV, HIV, HTLV, HCV and HPV viruses in induction of HCC, cervical cancer and lymphoma. **(17, 15, 4)**

Kalaitzakis and his colleagues approved that the risk of non-hepatic cancers increases with cirrhosis.[9] and HCV is a well-known cause of cirrhosis, so HCV infection may play a role in induction of non-hepatic cancer.

The current study suggests that HCV infection plays a role in carcinogenesis of the colon, and breast supported by detection of HCV proteins within the investigated tumor cells by IHC. Interestingly, it was established by Liao and his colleagues that IHC of HCV proteins has the upper hand to detect HCV in tissues than HCV PCR. This is due to the latter fragility and low level of viral expression in infected tissues. [14]

Hassanin and his colleague reported that IHC is comparable to RT-PCR in detection of HCV antigen in seropositive and seronegative persons.**(8)** which is matched with our results; as 100% of cases were stained with Cocktail antibody and 97% of them were positive for HCV core antibody. This indicates that IHC is a simple and commercially available method for detection of HCV virus in the tissues. The negativity of three

cases to HCV core antibody may be due to low viral load, which is also suggested by Paydas.[15]
The small sample size of the current study might be attributed to targeting only seropositive patients, and excluding seronegative patients but according to Elkashef and his colleagues seronegativity or occult HCV infection induce Non Hodgkin lymphoma and other extra hepatic malignancy.[5] Occult HCV infection is diagnosed by liver biopsy and low total protein and serum albumin.[5]
The Cocktail antibody is composed of three viral proteins including NS3 which indicate viral replication within the cell. This also may explain that all cases are positive for this marker.
Regarding NS4 non-structural HCV protein, Attallah and his colleagues reported that HCV RNA was detected in 143 out of 186 (77%) of Egyptian patients, 93% of them were positive for NS4 protein. They detected NS4 protein in patients with no detectable HCV RNA concentration [3] which is close to our results.
According to the present study, the cytoplasmic staining pattern of both IHC markers (HCV core protein and Cocktail) extend beyond the nuclear pattern, which is confirmed by IHC study performed by Kasprzak, et al., who also confirm the subcellular localization of Core protein and NS3 in cisternae of endoplasmin reticulum and mitochondria.[10]
Unlike our results, a study from Taiwan detected strong relation between HCV infection and colon cancer in patients below 45 years old [18] but in the present study the range of age of colon tumors was 35 to 85, and the cases above the age of sixty predominate. This discrepancy from our finding may be due to small number of our cases, few data are available about patients PCR positive HCV and including only seropositive patients.
In the present study, the grading of colon cancer was significantly correlated with scoring of HCV core protein, as most high- grade colon cancer cases had high H score. This means that HCV infection may play a role in carcinogenesis, differentiation and severity of the tumors but this is in contrast with the results of Kasprzak, who found that IHC expression of HCV core or NS3 didn't correlate with the grading of colon cancer. [11]
Su et al. found a relation between chronic HCV and occurrence of breast cancer in patient <50 years.[19] Although, a study done by Larrey, et al. mentioned that several cases of breast cancer have been discovered during the regular follow up of large cohort of patients chronically infected by HCV [13] but multiple studies failed to reveal any relation between HCV infection and breast cancer. (13, 1)

Although the clinical and pathological parameters of breast cancer cases in this study did not reveal any significant relationships with the H score of HCV core or Cocktail HCV proteins. This may be due to small sample size, including only seropositive patients and low rates of screening.
In addition, there was no available data about patients that had been treated from the HCV infection that is necessary for detection of these proteins by IHC.
Attallah et al. approved that HCV infection is not a powerful risk for breast cancer by comparing the HCV prevalence between breast cancer patients and others with benign breast diseases.[2] Additionally, Lam and his colleagues observed that HCV infection is associated with low risk of breast cancer.[12] But according to the current study, HCV infection is associated with high grades and lymph node metastasis within breast cancer cases.
In conclusion, although our results revealed no significant relationships between expression of HCV core and Cocktail antibodies, and most of clinic pathological parameters of colon and breast cancer, but most of our cases gave positive staining and this observation open the road in front of further studies on large sample size to detect the relationship of the HCV infection to non-hepatic solid tumors.

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